Chondrocytic differentiation of mesenchymal stem cells sequentially exposed to transforming growth factor-β1 in monolayer and insulin-like growth factor-I in a three-dimensional matrix

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Abstract

This study evaluated chondrogenesis of mesenchymal progenitor stem cells (MSCs) cultured initially under pre-confluent monolayer conditions exposed to transforming growth factor-β1 (TGF-β1), and subsequently in three-dimensional cultures containing insulin-like growth factor I (IGF-I). Bone marrow aspirates and chondrocytes were obtained from horses and cultured in monolayer with 0 or 5 ng of TGF-β1 per ml of medium for 6 days. TGF-β1 treated and untreated cultures were distributed to three-dimensional fibrin disks containing 0 or 100 ng of IGF-I per ml of medium to establish four treatment groups. After 13 days, cultures were assessed by toluidine blue staining, collagen types I and II in situ hybridization and immunohistochemistry, proteoglycan production by [^{35}S]-sulfate incorporation, and disk DNA content by fluorometry. Mesenchymal cells in monolayer cultures treated with TGF-β1 actively proliferated for the first 4 days, developed cellular rounding, and formed cell clusters. Treated MSC cultures had a two-fold increase in medium proteoglycan content. Pretreatment of MSCs with TGF-β1 followed by exposure of cells to IGF-1 in three-dimensional culture significantly increased the formation of markers of chondrocytic function including disk proteoglycan content and procollagen type II mRNA production. However, proteoglycan and procollagen type II production by MSC's remained lower than parallel chondrocyte cultures. MSC pretreatment with TGF-β1 without sequential IGF-I was less effective in initiating expression of markers of chondrogenesis. This study indicates that although MSC differentiation was less than complete when compared to mature chondrocytes, chondrogenesis was observed in IGF-I supplemented cultures, particularly when used in concert with TGF-β1 pretreatment. © 2001 Orthopaedic Research Society. Published by Elsevier Science Ltd. All rights reserved.

Introduction

Osteoarthritis is a common cause of disability and reduced quality of life among the elderly. Federal agencies project more than 60 million people will be affected by osteoarthritis (OA) by the year 2020 [1]. The high incidence of OA is due in part to the minimal intrinsic healing capacity of injured articular cartilage. Despite this, there has been significant progress in improving cartilage repair through transfer of allogenous or autogenous tissues, and through free cell transplantation procedures [4,6,9,23,40,50,52,57]. Cartilage resurfacing by transplantation of autogenous or allogenous chondrocytes, or through the use of mesen-chymal stem cells (MSCs), has several advantages over solid tissue transplantation or local debridement procedures [4,27,39,55]. However, allogenous chondrocytes carry an inherent risk of an immune reaction [13,28,34,44], and although the use of autogenous chondrocytes avoids this risk, the lack of a suitable donor site and the need for large cartilage samples limit autogenous chondrocyte applications.

Mesenchymal stem cells are undifferentiated pluripotential cells capable of differentiating into many cell types and may be a suitable autogenous cell source for articular cartilage repair [7]. In immature animals MSCs assist in cartilage repair through direct contributions to the cellular pool in healing cartilage defects [5]. However, in adults, MSCs are incapable of establishing a durable repair in lesions greater than 5 mm diameter, possibly as a result of diminished MSC population density or through reduced potential to form fully

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differentiated chondrocytes [10,30]. Further evaluation of methods to bolster the declining chondrogenic and metabolic capacities of MSCs from mature individuals would clearly assist in both intrinsic and transplanted cartilage repair mechanisms in adults.

Previous studies show MSCs derived from bone marrow and perichondrium have chondrogenic potential [7]. In vitro experiments show markers of chondrogenesis, such as aggrecan and collagen type II, are expressed in MSC cultures [3,32]. Furthermore, in vivo experiments show stem cells implanted into surgically-created osteochondral defects in rabbits are able to differentiate into chondrocytes and improve the quality of cartilaginous repair tissue [25,61]. The clinical advantages of using bone marrow-derived MSCs include the routine harvest of cells under local anesthesia which obviates the risk and cost of general anesthesia, the ability to avoid significant damage to articular donor sites, the capability of expanding cell numbers in vitro prior to re-implantation, and the lack of an immune reaction to the cell membrane [20]. Methods to stimulate proliferation and subsequent chondrogenic differentiation of MSCs are needed to further develop the use of cultured MSCs for articular defect repair.

In initial cultures, clonal mesenchymal cells have variable pluripotentiality. After proliferation, MSC differentiation occurs, guided by the immediate culture conditions. A number of growth factors, including FGF, TGF-β1, and IGF-I have been evaluated for their ability to enhance chondrogenesis of MSCs [32,36,63]. TGF-β1 has been shown to stimulate collagen type II and proteoglycan expression in MSCs [63]. In vitro, MSCs respond to TGF-β1 in a dose-dependent manner, with a concentration range of 0.1–5 ng/ml resulting in effective chondrocytic differentiation [62,63]. Furthermore, TGF-β1 can induce chondrogenic transformation with short exposure times and under culture conditions that stimulate high cell densities [22]. Repair of partial thickness articular defects in vivo was enhanced by TGF-β1 treatment, which induced MSC recruitment from the synovium [29]. By contrast, TGF-β1 reduces matrix protein expression in freshly isolated articular chondrocytes [19], but stimulates expression of collagen type II and proteoglycan in chronically cultured cells [60]. Exogenous IGF-I increases matrix proteoglycan and collagen type II deposition by chondrocytes in vitro and in vivo [17,21,37,43,48,59]. Studies of arthritic cartilage in man show an initial increase in IGF-I, which may be essential for enhancing the local repair mechanisms mediated by subchondral MSCs and local chondrocytes [41,51]. However, IGF-I is decreased in severe osteoarthritis, suggesting a decompensatory loss of extracellular matrix synthesis may be associated with propagation of osteoarthritis [11,54]. While TGF-β has been shown to enhance MSC chondrogenesis, IGF-I can also enhance extracellular matrix synthesis by MSCs, forming a potentially valuable dual stimulatory effect on intrinsic or transplanted MSC function [12,16,45,58].

Cell transplantation techniques for cartilage resurfacing have frequently employed naturally adhesive vehicles to provide a three dimensional milieu to maintain the chondrocyte phenotype and to stimulate matrix metabolism [27]. Vehicles described previously include fibrin, collagen, alginate, and hyaluronate [27,53]. Autogenous fibrin is easily obtained and provides a matrix for cellular immunoprotection and graft attachment to exposed subchondral bone [26,27]. Moreover, the ability of fibrin to polymerize from a mixture of fibrinogen and thrombin provides a versatile matrix that can be applied arthroscopically. Similar matrices may be used for MSC transplantation, but could also be engaged in a carrier function for growth factor delivery to stem cell pools, both within the vehicle and in the graft bed [47]. Several groups have proposed that MSCs are a viable alternative to fully differentiated articular chondrocytes as a cell source for articular grafting [25,61]. We hypothesized

MSCs
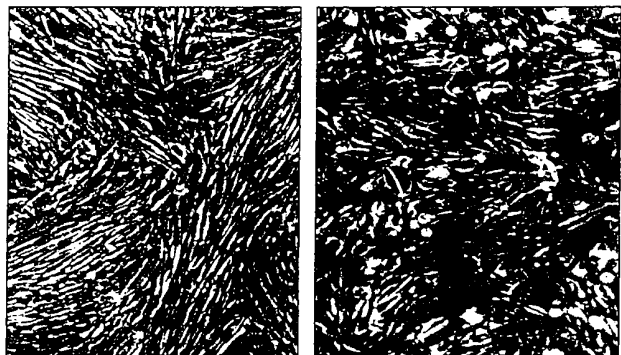
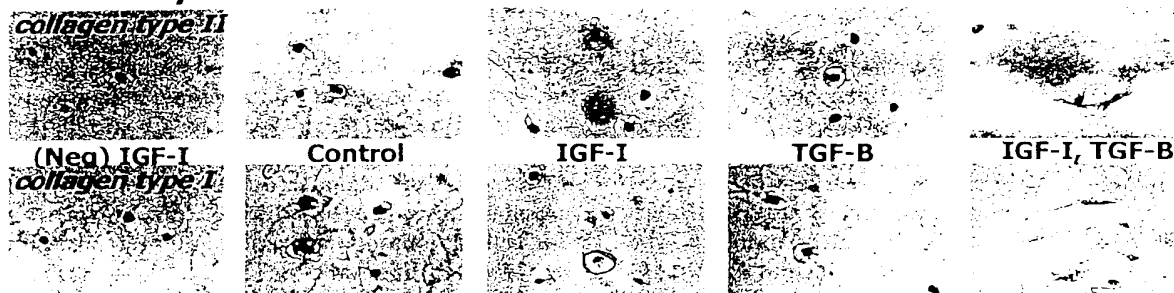## Chondrocytes
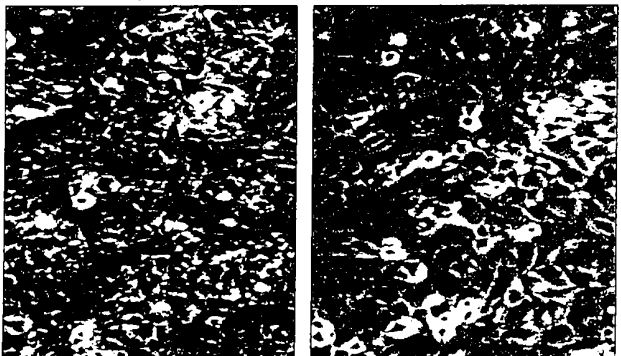
Control   **5 ng TGF-β1/ml**

Fig. 1. Phase-contrast photomicrographs of MSCs and chondrocytes in monolayer culture after 2 days of exposure to 0 or 5 ng of TGF-β1/ml. MSCs treated with TGF-1 have a rounded morphologic appearance compared to untreated controls. Chondrocytes show cellular enlargement with TGF-β1 treatment.

Chondrocytes

MSCs

Fig. 2. Collagen immunohistochemical reaction around mesenchymal stem cells (MSCs) and chondrocytes cultured in fibrin disks. MSCs show type-I collagen immunoreaction in all treatment groups. MSCs treated with insulin-like growth factor-I (IGF-I), with or without transforming growth factor-β1 (TGF-B) treatment in monolayer, contain areas of type-II collagen immunoreaction. MSC type-II collagen reaction was less profound than in parallel chondrocyte treatment groups. Negative procedural controls for IGF-I treated groups are provided [(Neg) IGF-I].

that MSC chondrogenesis could be enhanced by sequential TGF-β1 exposure in monolayer followed by IGF-I treatment in a three-dimensional fibrin matrix. Our objectives were to evaluate the chondrogenic effects of TGF-β1 on MSCs in monolayer culture, compare the responses of MSCs and mature articular chondrocytes to TGF-β1 and IGF-I, and to assess the effect of IGF-I on MSC metabolism in a three-dimensional culture.

Materials and methods

Cell culture

Mesenchymal cell harvest, isolation, and storage. Bone marrow aspirates were obtained aseptically from the sternum, tuber coxae, or proximal humerus of seven healthy horses ranging from 3 days to 2 years old (3 days, 2-, 3.5-, 10-, 12-, 17-, and 24-months of age – skeletally mature at 22-28 months). All horses were sedated, and the harvest sites aseptically prepared and infiltrated with local anesthetic. Bone marrow biopsy needles (Jamshidi, VWR Scientific, Bridgeport NJ) were used to aspirate bone marrow into 60 ml chilled syringes containing 8500 U of heparin per ml of Tyrode's solution (GIBCO-Life Technologies, Grand Island, NY). Four to eight 60 ml syringes were obtained from each animal. Thirty mls of aspirate was resuspended with 15 mls of Tyrode's solution and centrifuged twice at $300 \times g$ for 15 min to remove red blood cells. The pelleted cells were resuspended in 10 mls of medium (Ham's F12 medium supplemented with 10% fetal bovine serum, 50 μg of ascorbic acid/ml, 30 μg of σ-ketoglutaric acid/ml, 300 μg of L-glutamine/ml, 100 U of sodium penicillin/ml, 100 μg of streptomycin sulfate/ml, and 25 mM of HEPES buffer). The resuspended cells were cultured for 5 days at 5% CO_2, 90%

humidity, and 37°C in 75 cm² flasks. After the initial five days, the medium was changed every other day. To expand cell numbers, dense cell plaques were trypsinized and re-plated until they formed confluent monolayers, which were then trypsinized and passaged into duplicate 75 cm² flasks. Yield from each flask was 6-8 million cells. The resulting monolayer cultures appeared homogeneous and morphologically resembled cells used in previous studies of MSC propagation and transplantation [25,32,61]. Some cell heterogeneity may have persisted, despite the phenotypic similarities. After the second passage (total culture duration 12-17 days), confluent monolayers of mesenchymal cells were cryopreserved and stored in liquid nitrogen until cells from all the test animals were available to begin the experiments. The cryovials were then thawed in a 37°C water bath and immediately resuspended in 10 ml F12 medium. Cell number and viability were determined for each animal using the supravital stains fluorescein diacetate and propidium iodide, prior to combining all the MSCs. The MSC viability at the time of thawing ranged from 84.1% to 100%.

Chondrocyte harvest, isolation, and storage. Articular cartilage was harvested aseptically from the same or age matched foals. Harvested cartilage was placed in chilled balanced salt solution, weighed, diced, and digested in collagenase as previously described [49]. The chondrocytes were separated from any remaining matrix by filtration through four layers of sterile gauze and nylon mesh (44 μm). The filtered chondrocytes were counted, resuspended in freeze media, and stored in liquid nitrogen until required, using the same freeze and thaw protocols as for the MSCs.

Mesenchymal cell cultures. MSCs were combined, centrifuged, counted, and replated at 9.6×10^6 cells per 75 cm² culture flask. Ten flasks were cultured with serum-free Hams F-12 medium containing the additives previously described, and supplemented with 5 ng/ml TGF-β1, and 10 control flasks were cultured in medium without TGF-β1. The dose of TGF-β1 was based on optimal responses to 5 ng/ml TGF-β1 evident in earlier studies [62]. Cells were maintained in monolayer for 6 days, with exchange of medium (0 or 5 ng/ml TGF-β1) every other day. On day 6, monolayer cultures were trypsinized.
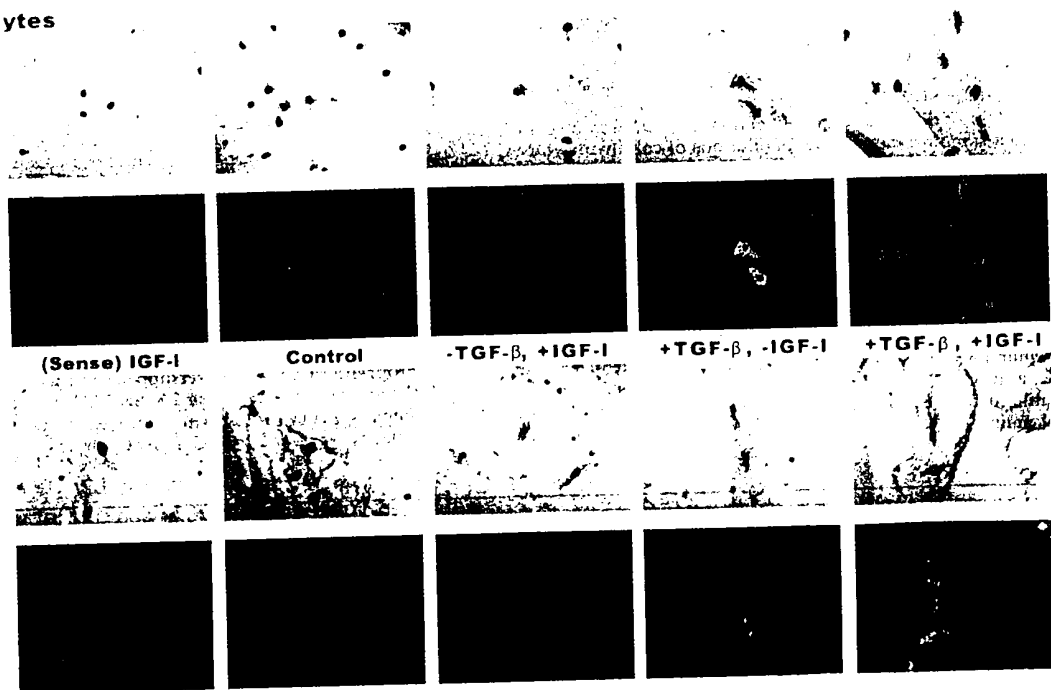
A

Collagen type I
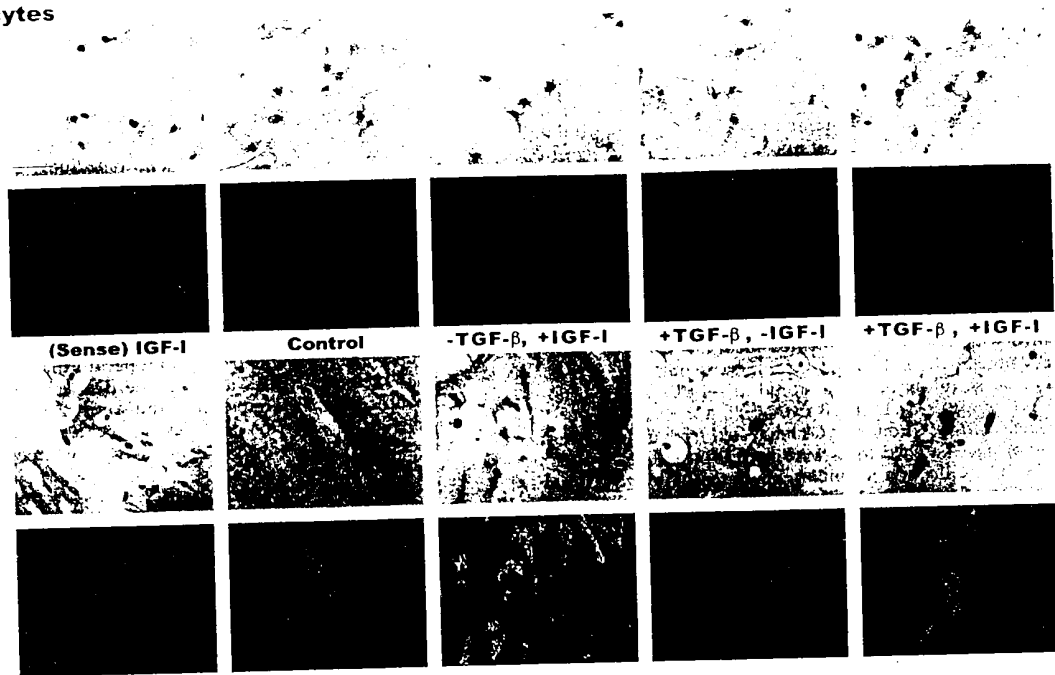
B

Collagen type II

counted, and pooled depending on previous TGF-β1 supplementation (0 or 5 ng/ml). The mesenchymal cell suspension from the 0 ng/ml and 5 ng/ml TGF-β1 cultures was divided into two aliquots from each, centrifuged, and the cell pellets resuspended in equine fibrinogen (200 mg/ml; Sigma Chemical, St. Louis, MO) to form a mixture containing 9.8 × 10⁶ cells/ml, 2 TIU/ml of aprotinin (Sigma), and either 0 or 200 ng/ml of IGF-I (Genentech, San Francisco, CA). Each fibrin disk was polymerized by combining 0.5 ml calcium activated bovine thrombin (Sigma) with 0.5 ml of the fibrinogen-cell suspension. The resulting three-dimensional fibrin disk contained 100 U of thrombin, 100 mg of fibrinogen, 4.9 × 10⁶ cells, 1 TIU of aprotinin and 0 or 100 ng of IGF-I, in a total culture volume of 1 ml. Disk thickness was 4 mm. The four treatment groups consisted of pretreatment with 0 or 5 ng/ml of TGF-β1 in monolayer followed by 0 or 100 ng/ml of IGF-I within the three-dimensional fibrin disk. The concentration of IGF-I selected was determined from the literature relevant to IGF-I effects on chondrocytes in fibrin cultures [17], and from preliminary studies of IGF-I on MSC function [15]. Disks were covered with 1.5 ml of serum-free medium supplemented with 0 or 100 ng/ml IGF-I. Disks containing IGF-I were supplemented with IGF-I supplemented medium. Fresh medium was added every other day. Exhausted medium was harvested, combined with 10% protease inhibitors, and stored at −70°C for glycosaminoglycan (GAG) analysis by DMMB dye-binding method.

Chondrocyte culture. Chondrocytes harvested from articular surfaces of the same or age-matched foals were used in a parallel culture protocol with MSCs, following a similar experimental design of TGF-β1 supplementation of monolayer cultures, and later separation to IGF-I treated or IGF-I untreated three-dimensional fibrin cultures.

Culture harvest. All fibrin disk experiments were terminated after 13 days in culture. Sixteen hours prior to harvest, a 2 mm cross-section of each fibrin disk was sliced from each well and placed in 4% paraformaldehyde at 4°C for in situ hybridization, immunohistochemistry, and histology. The remaining disk was radiolabeled with 20 μCi/ml of ³⁵[S]-sulfate in the medium. Sixteen hours later the disks were rinsed in protease inhibitors in water, frozen, lyophilized, weighed, digested (10 mg dry wt/ml) in 0.5% papain (Sigma) at 65°C for 24 h, and assayed for DNA content, total glycosaminoglycan (GAG) level, and incorporated disk ³⁵[S]-GAG.

Biochemical analyses

Total DNA analysis. DNA content of the papain digested fibrin disks was determined by combination with bisbenzimide (Hoechst 33258, Sigma) and fluorometric quantification [35]. DNA content was derived from a standard curve of serial dilutions of calf thymus DNA.

Hydroxyproline assay. Hydroxyproline content in cultures was assessed by use of reverse phase high-performance liquid chromatography (HPLC) of phenylisothiocyanate (PITC) derivatized samples [2]. Duplicate lyophilized samples were hydrolyzed in 6N HCl at 110°C for 22 h on an hydrolysis work station (Waters Division, Millipore, Millford, MA). The hydrolyzed samples were derivatized with PITC and 1 μg was analyzed by rpHPLC on a 30 cm C-18 reversed phase Pico-Tag™ column (Waters), eluted using a gradient of 6 to 31% acetonitrile in 140 mM sodium acetate with 0.05% triethylamine, pH 5.7. The column was maintained at 46°C, with a flow rate of 1 ml/min for the separation phase. Commercial software packages (Maxima, Waters) utilized for controlling and integration procedures, provided individual amino acid values from a standard curve of mixed amino

acids. Molar proportions of proline and hydroxyproline were used to derive collagen as a percentage of total protein.

Glycosaminoglycan (GAG) analysis. Exhausted medium (800 μl) was combined 1:1 with an equal volume of 0.5% papain and digested for 4 h at 65°C. Total glycosaminoglycan (GAG) content of the exhausted medium and the papain-digested disks was assayed from duplicate aliquots of papain-digested media (500 μl) and disk (50 μl). Each aliquot was combined with 2.5 ml of dimethylmethylene blue solution (in formate buffer) and optical density was determined at 525 nm. A standard curve was constructed using chondroitin-4-sulfate. Incorporation of ³⁵S into GAG in disk and medium was determined by alcian blue precipitation and scintillation. Aliquots of ³⁵S-labeled papain-digested disk or medium were placed in multiwell punch plates (PDVF plate, Millipore, Bedford, MA), precipitated with alcian blue, and counted by scintillation [38].

Histology, immunohistochemistry, and in situ hybridization

Histology. The 2 mm disk cross sections obtained prior to ³⁵[S]-sulfate labeling were placed in 4% paraformaldehyde for 24 h followed by immersion in PBS at 4°C. The disk sections were dehydrated in alcohol, embedded in paraffin, and sectioned at 6 μm. Routine staining using H&E, and toluidine blue histochemical reaction, were used to assess morphologic reaction and definition of pericellular proteoglycan deposition, respectively.

Collagen immunohistochemistry. Cross-sections were processed for collagen types I and II immunohistochemistry. The sections were exposed to testicular hyaluronidase (5 μg/ml) for 60 min at 37°C, followed by a nonspecific antibody block with normal goat serum for 20 min at 25°C. Endogenous peroxidase reactions were quenched with 0.5% H₂O₂ in methanol. Primary antibody, rat anti-bovine type II collagen (Michael Cremer, Veterans Administrative Hospital, Memphis, TN), was applied for 1 h at room temperature. Normal rat serum was used as a negative control on a serial section on each slide. A biotinylated secondary antibody (goat anti-rat) was applied for 20 min followed by streptavidin conjugated peroxidase to catalyze chromogen development of 3,3′-diaminobenzidine tetrahydrochloride. All slides were counterstained with Harris's hematoxylin. Collagen type I immunohistochemistry was processed similarly with a rabbit anti-equine type I antibody developed in our laboratory, and biotinylated secondary antibody (goat anti-rabbit). Non-immune rabbit serum was used for a negative control on each slide. Tendon and articular cartilage tissues for each antibody were also included in each procedure as a positive and negative control.

Collagen gene expression. In situ hybridization was performed with ³⁵S-UTP labeled riboprobes for equine collagen type α1 (I) and type II, using previously described techniques [17]. The riboprobes for equine collagen II were derived from a 209-bp cDNA region encoding exons 1 to 7 (excluding exon 2) of type II procollagen mRNA. The cDNA insert was cloned into pGEM-3zf (±) expression vector (Promega, Madison, WI). The [³⁵S]-UTP (Dupont-NEN, Boston, MA) labeled sense probe was transcribed by T7 RNA polymerase on a Bam HI linearized DNA template. The [³⁵S]-UTP labeled antisense probe was transcribed by SP6 polymerase on a EcoRI linearized DNA template. After deparaffinizing, the disk sections were covered with 15 μl of the appropriate riboprobe, coverslipped, and incubated at 43°C for 12 h. The slides were washed with 2 × SSC (30 mM sodium citrate-300 mM sodium chloride solution) to remove coverslips, followed by a low stringency wash of 2 × SSC at 50°C for 15 min and a high stringency

Fig. 3. Composite in situ hybridization photomicrographs showing cellular transcription of collagen types I and II after 13 day exposure to insulin-like growth factor-I (IGF-I). Two of the four groups of cells were exposed to transforming growth factor-β1 (+TGF-β), prior to three-dimensional culture and the addition of IGF-I (+IGF-I). Controls were untreated during monolayer and untreated during three-dimensional culture: (A) collagen type I gene expression. Brightfield (top panel) and darkfield (lower panel) photomicrographs show type I expression is prominent in mesenchymal stem cells (MSCs) and minimal in chondrocyte cultures. Collagen type I expression by MSCs is enhanced by IGF-I exposure, irrespective of prior TGF-β1 treatment. Serial sections on each slide were also probed with riboprobes generated from the sense DNA strand, and brightfield and darkfield photomicrographs of IGF-I treated cultures are included as examples of this negative control [(Sense) IGF-I]. Slides exposed for 14 days. Bar = 60 μm; (B) collagen type II gene expression. MSC cultures treated with TGF-β1 in monolayer followed by IGF-I show enhanced type II development compared to control cultures. Cultures exposed to IGF-I or TGF-β1 alone show lesser type II expression than that evident when both growth factors were used. Minimal type II collagen is evident in untreated controls. Chondrocyte cultures showed similar trends, however, all cultures including controls show a stronger cellular type II collagen reaction. Slides exposed for 10 days.

wash of 0.5 × SSC for 60 min. All sections were treated with 20 μg/ml of RnaseA prior to washing in 2 × SSC/50% formamide for 2 h. The slides were dehydrated through an alcohol series containing 300 mM ammonium acetate prior to dipping in NTB2 emulsion (Eastman Kodak Co, Rochester, NY), diluted 1.1 with 600 mM ammonium acetate. The emulsion covered slides were developed after 14 days, counterstained with hematoxylin, and coverslipped.

Statistical analysis

Statistical significance between groups and within groups was determined for monolayer cell number, total DNA content (μg/mg dry weight), total disk glycosaminoglycan content (μg GAG/μg DNA), disk ^{35}[S]-glycosaminoglycan (log [cpm/μg DNA]), medium ^{35}[S]-glycosaminoglycan (log [cpm/μg DNA]), and total medium GAG (μg GAG/μg DNA) using a one-way analysis of variance and Bonferroni's post hoc test. A level of $P < 0.05$ was accepted as significant. The entire study was repeated once to confirm the original data.

Results

Morphology

Monolayer culture morphology. Under control conditions, MSC monolayer cultures formed multiple swirls and established a monolayer within 48 h. MSCs exposed to 5 ng of TGF-β1/ml proliferated and showed cellular rounding with cell clusters after 2 days (Fig. 1). Cell detachment along the edges of the flasks became apparent after 3 days of TGF-β1 exposure, and by 5 days of treatment MSCs in several areas had detached in sheets of viable appearing cells. Chondrocytes exposed to TGF-β1 showed cellular enlargement with small areas of cellular detachment when compared to controls.

Three-dimensional culture morphology. Cell shape in all treatment groups varied according to depth within the disk. Cells toward the base of the disks were predominantly rounded, while cells toward the surface of the disk exhibited a flattened morphology. Both MSCs and chondrocytes in TGF-β1-pretreated discs were organized in multicellular clusters, whereas cells in the control discs remained separate. IGF-I treated MSCs had a more uniform distribution of cells, while IGF-I groups pretreated with TGF-1 had multicellular clusters.

Histochemistry and immunohistochemistry. Pericellular toluidine blue metachromatic staining consistent with proteoglycan production developed in all groups. Staining was more evident toward the surface of the disks, and was more intense around chondrocytes than around MSCs. IGF-I treatment increased metachromatic staining in both MSC- and chondrocyte-laden disks. Additionally, collagen type II deposition was evident in IGF-I treated mesenchymal three-dimensional cell cultures (Fig. 2). All chondrocyte cultures had collagen type II expression, which was most prominent in the IGF-I-treated cultures. Collagen type II expression was markedly increased in chondrocyte cultures compared to MSC cultures. Collagen type I was expressed in

all MSC treatment groups, whereas type I expression in chondrocyte cultures was minimal. In both MSC and chondrocyte cultures, IGF-I increased collagen type I expression above controls. Both collagen type I and collagen type II expression was increased towards the surface of the disks.

In situ hybridization. Type I collagen mRNA was evident in sections from all MSC culture treatment groups (Fig. 3(A)), and was increased by IGF-I exposure. Type I collagen expression in chondrocytes was minimal in all treatment groups, and considerably less than the pronounced expression evident in MSC cultures. Collagen type II message was evident in MSCs pretreated with TGF-β1, and to a lesser extent in MSC cultures exposed to IGF-I without prior TGF-β1 (Fig. 3(B)). MSC controls showed minimal evidence of collagen type II message. Chondrocytes had increased collagen type II message with IGF-I and/or TGF-β1 treatment, compared to untreated controls.

Monolayer and three-dimensional cell populations

Monolayer cell numbers. Based on cell counts after 6 days, TGF-β1 treatment did not stimulate mitogenesis of the monolayer cultures. Mesenchymal stem cells exposed to TGF-β1 had a significant decline in cell numbers (36% of the initial seeding density), while controls had a 33% increase (Fig. 4). Chondrocytes exposed to 5 ng of TGF-β1/ml had a 32% increase in cell numbers
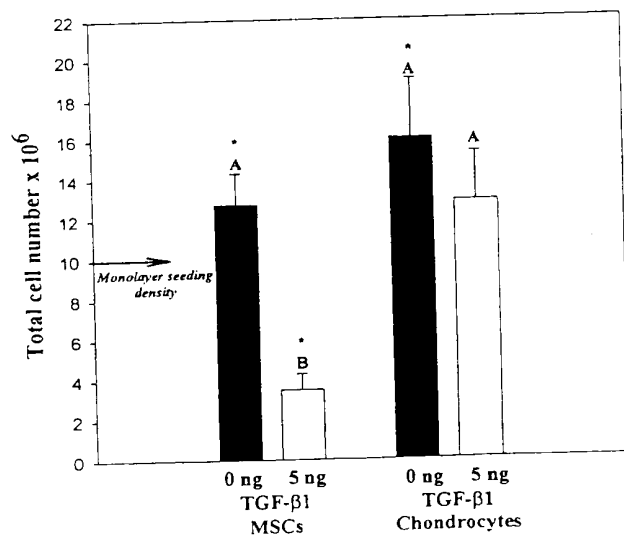
Fig. 4. Total numbers of mesenchymal stem cells (MSCs) and chondrocytes in monolayer cultures at the time of transfer to three-dimensional matrix. Data represent mean ± S.E. of cell number ×10⁶ per culture flask. Asterisks represent significant cell number differences compared to initial seeding density. Within each cell type, differing letters indicate significant differences due to TGF-β1 treatment at $P \leqslant 0.05$.

over the initial seeding density compared to a 67% increase in untreated controls.

Disk DNA content. Total DNA content was assayed on day 13 and was normalized to dry weight of the disks. IGF-I treatment significantly increased ($P = 0.048$) DNA content of MSCs when compared to controls (Table 1). No other significant changes were evident. Total DNA content of MSCs and chondrocytes in three-dimensional culture was similar.

Biochemical assays

Hydroxyproline content. Hydroxyproline levels (collagen) of chondrocyte cultures treated with IGF-I were significantly increased ($P = 0.0063$) compared to untreated controls (Table 2). MSCs showed similar trends for IGF-I induced collagen deposition.

Glycosaminoglycan assays. Medium GAG content in MSC and chondrocyte monolayer cultures was significantly (1.9–3.3-fold) increased above controls by TGF-β1 treatment in 6 day cultures (Fig. 5(A) and (B)). Total medium GAG content of MSCs was approximately one-half that of the chondrocyte culture medium GAG content. Glycosaminoglycan content of medium from MSC fibrin disk cultures showed a significant ($P \leqslant 0.05$) increase with IGF-I treatment compared to untreated controls on day 10 (Table 3). Glycosaminoglycan content of MSC cultures in control and IGF-I treated groups was significantly ($P < 0.001$) increased on all days compared to day 2. Medium GAG from chondrocyte fibrin disks showed significant ($P < 0.02$) increases on days 8, 10, and 12 with both IGF-I and TGF-β1 treatment. Medium GAG was most profoundly in-

creased in MSCs and chondrocytes pretreated with TGF-β1 prior to IGF-I exposure. Incorporated medium [35][S]-GAG on day 13 showed no significant trends in MSC ($P = 0.13$) or chondrocyte ($P = 0.13$) treated cultures.

Total disk GAG of MSC cultures was significantly ($P = 0.012$) increased with TGF-β1 pretreated cells exposed to IGF-I (Fig. 6(A) and (B)). All growth factor treatments significantly ($P = 0.0063$) increased chondrocyte disk GAG content, compared to controls. Accumulation of [35][S]-GAG was significantly increased in chondrocyte-laden disks treated with IGF-I (Fig. 7(A) and (B)). MSCs showed similar, but not statistically significant trends ($P = 0.14$). Overall, total GAG and [35][S]-GAG content of MSCs was lower (20–38% of disk GAG) than parallel chondrocyte GAG production.

Discussion

Combination of TGF-β1 pretreatment of MSCs followed by IGF-I exposure in three-dimensional culture resulted in significant chondrogenic transformation. Markers of chondrocytic function such as phenotypic condensation, collagen type II mRNA and protein production, and PG accumulation were maximal in TGF-β1 pretreated cultures exposed to IGF-I for 13 days. IGF-I alone induced chondrogenesis to a lesser degree. Despite these changes, chondrocyte phenotypic expression of MSCs was significantly less than articular chondrocytes cultured under similar conditions to provide positive controls.

Table 1
Disk DNA content of MSC and chondrocyte cultures at termination on day 13[a]

	Day 0 Control	−TGF-β1 −IGF-I	−TGF-β1 +IGF-I	+TGF-β1 −IGF-I	+TGF-β1 +IGF-I
MSC	N/A	4.65 ± 0.25	5.88 ± 0.34*	5.09 ± 0.15	4.99 ± 0.30
Chondrocyte	5.75 ± 0.38	7.67 ± 0.95	6.87 ± 0.66	5.41 ± 0.40	6.99 ± 0.90

[a] Cultures were pre-treated with TGF-β1 in monolayer (+TGF-β1) or cultured as untreated controls (−TGF-β1), and then divided for subsequent three-dimensional culture in IGF-I (+IGF-I) or untreated controls (−IGF-I). Data represents mean ± SEM of μg DNA/mg of dry weight disk using the bisbenzimide (Hoechst 33258) fluorometric assay. Asterisks represent significant difference within MSC and chondrocyte cultures due to treatment. Due to insufficient MSC cell numbers a day 0 control was not performed (N/A).

Table 2
Hydroxyproline content of MSC and chondrocyte laden disks at termination on day 13[a]

	−TGF-β1 −IGF-I	−TGF-β1 +IGF-I	+TGF-β1 −IGF-I	+TGF-β1 +IGF-I
MSC	137.4 ± 23.9[A]	171.5 ± 19.5[A]	158.5 ± 13.7[A]	118.6 ± 23.9[A]
Chondrocyte	299.8 ± 61.5[A]	489.6 ± 69.9[B]	246.1 ± 30.2[A]	219.8 ± 13.2[A]

[a] Cultures were pre-treated with TGF-β1 in monolayer (+TGF-β1) or cultured as untreated controls (−TGF-β1), and then divided for subsequent three-dimensional culture in IGF-I (+IGF-I) or untreated controls (−IGF-I). Hydroxyproline was determined by precolumn PITC derivatization of hydrolyzed samples and Pico-Tag™ reverse phase high-performance liquid chromatography separation techniques. Data represents mean ± SEM of pg hydroxyproline/μg of dry weight disk. Different letters represent statistical significance due to growth factor treatment within each cell type. No statistical comparison was done for data comparing hydroxyproline content in MSC and chondrocyte cultures treated similarly.
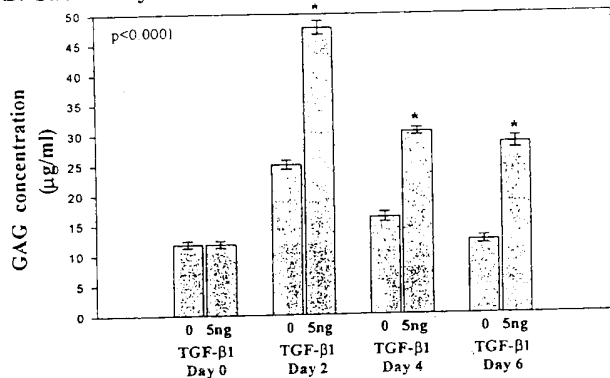
A. MSCs
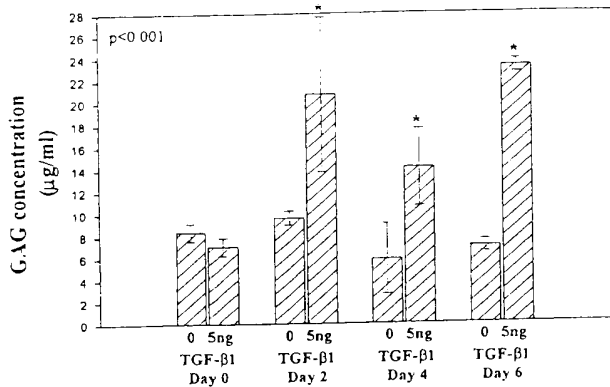
B. Chondrocytes

Fig. 5. Glycosaminoglycan (GAG) content of medium from, (A) mesenchymal stem cells (MSCs), and (B) chondrocyte monolayer cultures. Medium was papain digested and mixed with DMMB prior to absorbance detection by spectrophotometry. Data represents mean ± S.E. Asterisks represent significant difference due to transforming growth factor-β1 (TGF-β1) treatment within each time point.

Morphologic changes observed in monolayer cultures after 2 and 3 days of TGF-β1 exposure were consistent with previous descriptions of progenitor cell proliferation and chondrocytic differentiation [56]. Continued cell to cell attachment with cell to surface detachment were evident, and these findings are consistent with descriptions of MSC condensation and chondrogenesis [32,64]. Previous research using higher cell density cultures has shown enhanced MSC chondrogenic potential with low doses of TGF-β1 (0.1–1.0 ng/ml) for up to 12 days [33], or similar concentrations (5 ng/ml) for shorter periods (4 days) at similar densities (1.6×10^5 cells/cm^2) to those used in this study [62]. The eventual decline in cell numbers in the current study is consistent with results seen by others using high concentrations of TGF-β1 [31]. Morphologic appearance of cultures on days 2–4 suggested TGF-β1 induced cell proliferation and the final quantitative analysis on day-6 may have missed an important mitogenic aspect of TGF-β1 on MSCs. With the paucity of information concerning the effects of TGF-β1 concentration on bone-marrow derived progenitor cell chondrogenesis, a dose of 5 ng/ml was used here based on positive results of previous monolayer experiments [62]. A decline in cell numbers may also reflect a loss of metabolically active viable cells to the medium. This is consistent with the continued high PG accumulation in TGF-β1 treated cultures on day 6 compared to days 2 and 4 (Fig. 5). Cumulative information from these and previous experiments suggests shorter exposure times to TGF-β1 or use of lower concentrations may enhance chondrogenic transformation without the significant decline in cell numbers seen here by day 6. However, capture and use of detached cells in long-term exposure protocols may still be useful in deriving chondrogenic cells for transplant. Exposure to

Table 3
Medium GAG content of MSC and chondrocyte fibrin disk cultures[a]

	−TGF-β1 −IGF-I	−TGF-β1 +IGF-I	+TGF-β1 −IGF-I	+TGF-β1 +IGF-I
MSCs				
Day 2	6.12 ± 0.65	6.38 ± 0.22	6.49 ± 0.27	6.63 ± 0.38
Day 4	6.74 ± 0.32	6.46 ± 0.29	7.09 ± 0.10	7.14 ± 0.18
Day 6	7.02 ± 0.23	7.35 ± 0.50*	7.66 ± 0.18	7.31 ± 0.12
Day 8	7.69 ± 0.26	7.80 ± 0.53*	7.00 ± 0.41	11.78 ± 4.41
Day 10	8.12 ± 0.39*AB	8.40 ± 0.79**AB	6.93 ± 0.28A	10.00 ± 0.86B
Day 12	8.47 ± 0.38*	9.50 ± 1.11**	7.18 ± 0.28	9.36 ± 0.47
Chondrocytes				
Day 2	6.88 ± 0.24	6.35 ± 0.56	6.10 ± 0.13	6.52 ± 0.57
Day 4	7.41 ± 0.22	6.73 ± 0.55	8.81 ± 0.72	9.20 ± 1.83
Day 6	9.89 ± 0.24*	9.46 ± 1.11	12.78 ± 0.99*	14.64 ± 2.49*
Day 8	12.10 ± 0.52**A	13.90 ± 1.98*A	14.31 ± 1.70**A	21.90 ± 1.52**B
Day 10	14.18 ± 0.69***A	13.65 ± 1.92AB	13.75 ± 1.47*A	20.72 ± 2.15**B
Day 12	14.26 ± 0.25***AB	14.54 ± 1.56**AB	13.34 ± 1.30*A	20.01 ± 0.89**B

[a] Cultures were pre-treated with TGF-β1 in monolayer (+TGF-β1) or cultured as untreated controls (−TGF-β1), and then divided for subsequent three-dimensional culture in IGF-I (+IGF-I) or untreated controls (−IGF-I). Data represents mean ± SEM of GAG µg/ml of medium. Letters represent statistical significance within one time point due to growth factor treatment. Single and multiple asterisks represent significant increases in GAG content over time, within each treatment, compared to day 2.

TGF-β1 for as little as 30 min provides a chondrogenic stimulus to periosteal derived stem cells [42], and may provide a more suitable and certainly expeditious method to derive phenotypically transformed cells.

The increased proteoglycan production of MSC monolayer cultures supplemented with TGF-β1 correlates with later collagen type II expression data from the three-dimensional cultures, and further supports defined early chondrogenesis. Medium from subsequent three-dimensional cultures, without follow-up growth factor exposure, showed attenuated proteoglycan production, whereas treatment with IGF-I resulted in maximal proteoglycan content in both medium and disks. Additional IGF-I treatment of MSCs pretreated with TGF-β1 enhanced collagen type II gene expression and collagen translation. This data is consistent with previous

studies where IGF-I was shown to enhance extracellular matrix protein synthesis, proteoglycan production, and mitosis of both chondrocytes and MSCs [16,17].

Histologically, the spatial predominance of proteoglycan production was at the surface of the disk, suggesting that IGF-I in the medium or other components of the medium, had an impact on the cellular response. Although IGF-I laden fibrin disks elute IGF-I for a period of up to three weeks [14], the distribution of cellular response on histologic sections suggests that IGF-I in the medium had considerable influence on cell anabolism. While chondrocytes had a more dramatic response to IGF-I and/or TGF-β1 treatment, the gradient of cellular responses throughout the fibrin disks was similar. In vivo it may be more relevant to supplement synovial fluid with IGF-I in addition to IGF-I supplementation of the fibrin graft vehicles. Techniques for articular IGF-I enhancement are under evaluation,
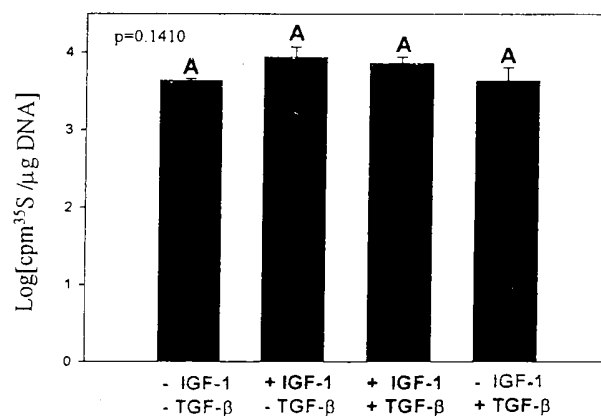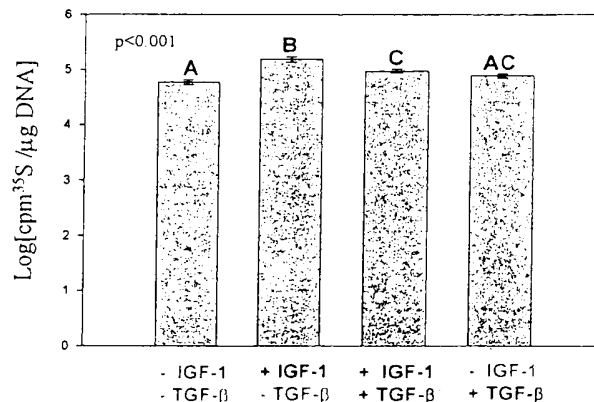
A. MSC
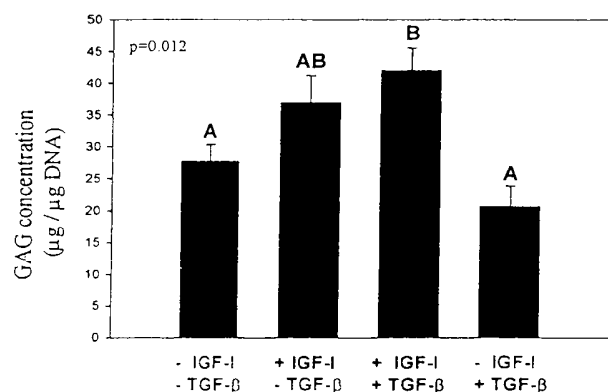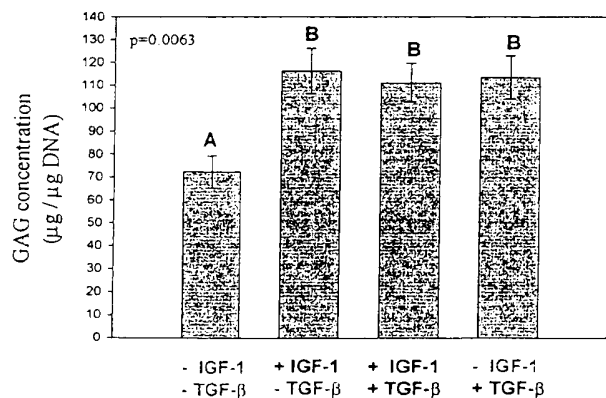
B. Chondrocytes

Fig. 6. Glycosaminoglycan (GAG) content of (A) mesenchymal stem cells (MSC), and (B) chondrocyte laden fibrin disks after 13 days of insulin-like growth factor-I (IGF-I) treatment (+IGF-I) of transforming growth factor-β1 pre-treated (+TGF-β) or TGF-β1 untreated (-TGF-β) cultures. Fibrin disks were digested with papain and assayed by DMMB dye binding. Data are normalized to DNA content and are presented as mean ± S.E. Differing letters represent a statistical significance between treatment groups.

A. MSCs

B. Chondrocytes

Fig. 7. Disk ^{35}S-[GAG] accumulation from day-13 mesenchymal stem cell (MSC) and chondrocyte cultures. Data are log transformed and represent the mean ± S.E. for each treatment group. Differing letters represent a statistical significance between treatment groups.

including IGF-I gene therapy for chondrocyte transfection [46], or exogenous IGF-I supplementation [18,47].

The increase in disk PG and collagen type II synthesis of chondrocytes treated with TGF-β1 is consistent with chondrocyte responses to TGF-β1 after long-term monolayer culture [24,60]. However, TGF-β1 supplementation of freshly isolated chondrocytes has an inhibitory effect on PG and collagen type II production [19,60]. These apparently disparate findings have been attributed to a culture induced modification of the type II TGF-β1 receptor, resulting in a receptor with reduced molecular weight compared to freshly isolated chondrocytes [24,60]. Ligand binding to these modified type II receptors is thought to continue, and formation of heteromeric complexes with the type I receptor and subsequent signal transduction are also maintained. Despite this, several explanations could account for an apparent increase in matrix synthesis in chronic cultures exposed to TGF-β1. Formation of homomeric interactions between smaller type II receptors may induce signal transduction without the need for type I receptor interaction [8], possibly using a different SMAD cytoplasmic domain cascade. Alternatively, the smaller type II receptor may bind ligand and form heteromeric complexes with type I receptors more effectively than in freshly isolated chondrocytes, enhancing the signal transduction through the normal type I serine/threonine kinase pathway. Confirmation of either mechanism is outside of the scope of this paper. Nevertheless, results of TGF-β1 supplementation in this experiment clearly support the possibility of type II receptor modification, with early PG increases and later evidence of collagen type II production in three-dimensional culture. These effects become especially relevant in the preparation of products for articular resurfacing, and conceivably more closely characterize the response of cells to TGF-β1 in the culture period prior to transplant. A similar temporal modification in stem cell TGF-β1 receptor size has not been documented, to the authors' knowledge.

Although MSC differentiation was incomplete when compared to mature chondrocytes, partial chondrogenesis was observed in IGF-I supplemented cultures, with or without TGF-β1 pretreatment. MSC pretreatment with 5 ng TGF-β1/ml resulted in cell proliferation initially, but later cell detachment, which reduced the beneficial effects of TGF-β1 for expanding cell numbers in monolayer or increasing markers of chondrogenesis. Given this data, the use of TGF-β1 as a stand-alone supplement to enhance chondrogenesis of MSC's has limited value. However, IGF-I may be useful as adjunct therapy to TGF-β1 for MSCs being cultured for later transplantation to articular defects, or as an exogenous growth factor for incorporation in transplantation vehicles.

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